1. Full title

Circulating human leucine-rich α-2-glycoprotein 1 mRNA and protein levels to detect acute appendicitis in patients with acute abdominal pain

2. Running head

Risk-assessment tool for abdominal pain in the Emergency Department

3: Authors

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6. Key words: abdominal pain; acute abdomen; acute appendicitis; diagnosis; mRNA

7. Previous presentation

1. **Rainer TH**, Leung LY, Chan CPY, Cheng NM, Lai PBS, Cheung YS, Graham CA. Add-On LRG1 Tests For Improving The Prediction Of Acute Appendicitis In Emergency Department Patients With Acute Abdominal Pain: Prospective Observational Study
At: 16th International Conference of Emergency Medicine. Organized by the African College of Emergency Medicine. Cape Town. 18 – 21 April 2016

2. Leung LY, **Rainer TH**, Chan CPY, Leung YK, Lai PBS, Cheung YS, Graham CA. Circulating leucine-rich α-2-glycoprotein 1 to detect acute appendicitis in patients with acute abdominal pain. *Conference Programme and Abstracts 2014; C278: 178*

At: 15th International Conference of Emergency Medicine. Organized by the Hong Kong College of Emergency Medicine. Hong Kong. 11 – 14 June 2014

8. List of abbreviations: AA: acute appendicitis; CT-computed tomography; LRG1: Leucine-rich-2-glycoprotein;.

9. Human gene

Glyceraldehyde 3-phosphate dehydrogenase: GAPDH

Leucine-rich alpha-2-glycoprotein 1: LRG1

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Abstract

Background: Elevated levels of circulating plasma and urine leucine-rich-2-glycoprotein-1 (LRG1) protein has been found in patients with acute appendicitis (AA) and may be useful for diagnosis. This study aimed to investigate whether combined tests including circulating LRG1 mRNA levels improves the early diagnosis of AA.

Methods: Between December 2011 and October 2012, a prospective study was conducted on patients aged 18 years or older presenting to the ED with acute abdominal pain (<7 days of symptom onset). Levels of whole blood LRG1 mRNA levels and plasma LRG1 protein taken from these patients within 24 hours of arrival (mean 12.4h) were analyzed. The primary outcome was AA.

Results: Eighty-four patients (40 (47.6%) with AA and 44 (52.4%) without AA; mean age 35 years; 41.6% males) were recruited. Median whole blood LRG1 mRNA and plasma LRG1 levels were higher in AA patients than in non-AA Of 40 AA patients, 13 (32.5%) were diagnosed as complicated AA, and had median LRG1 mRNA level higher than in patients with complicated AA In ROC analysis of LRG1 mRNA (normalized to GAPDH), LRG1 protein and Alvarado score for discriminating AA and non-AA, the areas under the curve (AUC) were 0.723, 0.742 and 0.805 respectively. The combination of normalized LRG1 mRNA, LRG1 protein and Alvarado score demonstrated the largest AUC (0.845). ***Conclusion:*** A combination of modified whole blood LRG1 mRNA levels, serum LRGI protein and Alvarado score at the ED may be useful to diagnose simple and complicated AA from other causes of abdominal pain..

Introduction

Acute appendicitis (AA) is a common life-threatening abdominal emergency, which in 2010, claimed 34800 deaths worldwide [1]. In the USA simple AA accounts for an average of 1.8 days in hospital, perforated AA accounts for 5.2 hospital days [2] and the incidence is increasing [3]. AA is commonly diagnosed based on clinical history, physical examination, simple laboratory tests and imaging [4-7], including the Alvarado score) [8, 9], white cell count or C-reactive protein [10], urinary 5-hydroxyindoleacetic acid (5-HIAA) [11]), and ultrasonography , CT and MRI [12-14] (see Appendix for a summary). The gold standard is CT imaging but this is not always available, and its radiation carries some cancer risk especially in the young. This has led some to search for alternative pathways for accurately diagnosing AA [7].

Circulating biomarkers have the potential to improve the diagnostic accuracy of AA in cases where utilizing CT or MRI would be inappropriate, delayed or unavailable. Leucine-rich α-2-glycoprotein-1 (LRG1) belongs to the leucine-rich repeat (LRR) family of proteins, many of which are involved in protein-protein interaction, signal transduction, and cell adhesion [15]. The biological function of LRG1 is unclear, but recently studies have demonstrated that LRG1 is expressed during granulocyte differentiation [16] and required for pathological angiogenesis [17]. Using a proteomic approach, LRG1 has recently been identified as a specific marker of AA [18,19]. High expressions of LRG1 protein have been found in the inflammed appendices of patients with AA, and increases in its level have been observed in urine and plasma of children with AA [18-20]. Its concentration correlated with histological severity of appendicitis [19-20]. A new diagnostic marker of specific LRG1 peptides using selected ion monitoring mass spectrometry has been developed, and superior diagnostic performance (AUC: 0.98) has been

93 demonstrated in the urine of children with AA. However, over 24 hours are required to detect
94 urine LRG1 using this method, which limits its application in emergency settings. A commercial
95 ELISA for LRG1 has also become available, and with shorter processing times than mass
96 spectrometry. However, immunoassay interference resulted in inadequate performance for
97 clinical use [19-20].
98
99 Although previous studies have studied changes in protein levels, such levels are dependent upon
100 the upstream expression of LRG1 mRNA which encodes LRG1 protein. Nucleic acids are well
101 regarded as early markers of acute illness and injury [21-36], and we have previously
102 demonstrated a potential clinical role for plasma DNA as a prognostic marker in patients with
103 acute abdominal pain [21].
104
105 In adult patients aged over 18 years presenting to an emergency department with acute
106 abdominal pain, what is the add-on diagnostic and risk-stratification value of circulating levels of
107 LRG1 and LRG1 mRNA in patients with AA? We hypothesise that there are significant
108 differences in levels of circulating LRG1 and LRG1 mRNA between patients with AA, and those
109 patients without AA, and that there will be a positive correlation between circulating levels and
110 the severity of appendicitis. Thus the aims of this study were (1) to investigate the diagnostic
111 value of plasma LRG1 and whole blood LRG1 mRNA level in patients with suspected AA, and
112 (2) to elucidate the correlation between whole blood LRG1 mRNA and histological severity of
113 appendicitis, and (3) to investigate early temporal relationships in circulating LRGI and LRGI
114 mRNA in patients with acute abdominal pain. This may enable the development of novel protein

and mRNA-based blood markers or combinations to improve the diagnostic accuracy of simple and complicated AA.

Materials and methods

Subjects and data collections

Approval was obtained from Institutional Review Board of the Chinese University of Hong Kong to conduct this prospective study (CREC 2015.710). Written consent was obtained either from the patient or a relative in all cases.

Eligible patients included those aged 18 years and above, presenting to the Emergency Department of the Prince of Wales Hospital, Hong Kong, with abdominal pain of less than 7 days duration. Thirty-one healthy volunteers matched for mean age and sex were recruited. Final diagnosis was determined by the presence or absence of appendicitis on gross and histologic examination.

Inclusion and exclusion criteria

Patients aged 18 or above presenting to the ED with acute abdominal pain of likely surgical cause within 7 days of symptom onset were recruited. Patients were excluded if they were below 18 years of age, lack of consent, pregnant, had external blunt or penetrating trauma (due to an external force associated with a motor vehicle crash, fall or assault etc.), had known non-surgical causes for abdominal pain such as diabetic ketoacidosis, urinary tract infection, gastro-esophageal reflux, or indigestion (dyspepsia), had chronic medical conditions (e.g. inflammatory bowel disease, cancer, sickle cell anemia), or were taking chronic anti-inflammatory medications.

Definition

Acute abdominal pain was defined as pain occurring within 7 days of onset and in an area extending below the lower ribs, above the inguinal line and between the mid-axillary lines.

Acute appendicitis (AA) was defined as the presence of transmural inflammation of appendix or the presence of pus in the lumen of the appendix [23].

Acute appendicitis like syndrome (AALS) is usually characterized by clinical symptoms and physical examination. Clinical symptoms were classified as typical and atypical. Typical appendicitis usually included abdominal pain beginning in the region of the umbilicus for several hours, associated with anorexia, nausea or vomiting. The pain was then localized in the right lower quadrant, where tenderness developed. Atypical appendicitis lacked this typical progression and may include pain in the right lower quadrant as an initial symptom. Atypical appendicitis often requires ultrasound scan and/or CT scan to assist diagnosis.

The Alvarado score is also used for AA diagnosis [24]. The score has 6 clinical items (based on clinical symptoms and physical examination) and 2 laboratory measurements with a total of 10 points. A score below 5 is strongly against a diagnosis of appendicitis, while a score of 7 or more is strongly predictive of acute appendicitis.

Healthy controls were defined as age- and sex-matched volunteers with no history of recent acute illness within 3 months, chronic illness, smoking or medication.

Histologic severity of appendicitis was classified as having no inflammatory features (normal), foci of neutrophilic infiltration in the wall or mucosa (focal), scattered transmural infiltration (mild), dense transmural infiltration with tissue distortion (moderate), or dense transmural infiltration with tissue necrosis or wall perforation (severe) [8].

Data collection and measurable parameters

Using a standardized protocol, an English- and Cantonese-speaking research assistant collected demographic and previous medical data including age, sex, symptom onset time, time between sample collection and operative care, medical history (e.g. abdominal pain, seizures, hypertension, diabetes mellitus, ischaemic heart disease, atrial fibrillation, hyperlipidaemia, smoking etc.) and current medication.

Preparation of plasma and RNA extraction

A 10 ml venous blood was taken by standard venipuncture and collected into EDTA-tubes. Whole blood was collected and stored in TrizolLS (Invitrogen) at -80ºC for further analysis. Plasma was collected after centrifugation and stored at -80ºC for further analysis of LRG1 protein level. Total RNA was extracted from 400 ul whole blood and has been previously described [25].

One-step RT-qPCR for LRG1 mRNA and GAPDH mRNA

One-step real-time RT-qPCR was used for measuring the LRG1 mRNA concentrations in the whole blood RNA samples, based on previously reported methods [25]. The RT-qPCR assay for LRG1 was developed and optimized. The calibration curve for LRG1 mRNA quantification was prepared by assaying serial dilutions of HPLC-purified single-stranded synthetic DNA oligonucleotides (Sigma) specifying a 77-bp LRG1 amplicon, with concentrations ranging from 1×10^7 copies to 1×10^1 copies. The amplification primers were LRG1F (5'- ACTGCAACCCGCTTAACA -3') and LRG1R (5'- TCCCAAAGTGCTGGGATTAC -3'),

and the dual-labeled fluorescent probe was LRG1P [5'-(FAM) AATAATCCTGCCTTTGGCCGGGT (TAMRA)- 3', where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine]. For normalization, reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was measured and the assay for GAPDH has also been well established and described [25]. The concentration of LRG1 and GAPDH mRNA in the whole blood sample of patients and healthy controls were measured in duplicate.

ELISA for LRG1 protein analysis

Plasma LRG1 was quantified by human LRG assay (IBL, Fujioka, Japan) according to manufacturer's protocols. All samples and reagents were brought to room temperature 30 minutes before use. The level of LRG1 protein in plasma of healthy controls and patients were measured in duplicate.

Outcome measures

The primary outcome was the presence or absence of AA. The secondary outcome was the severity of appendicitis.

Statistical analysis

Descriptive statistics and data comparison tests (chi-squared, Fisher exact, Mann-Whitney, Kruskal-Wallis tests), Receiver Operating Characteristic (ROC) analysis, logistic regression, as well as diagnostic strength were carried out using MedCalc12.3 software (MedCalc Software bvba).

Results

Baseline characteristics

Between 14th December 2011 and 21st October 2012, 84 patients (40 (47.6%) with AA and 44 (52.4%) without AA; median age 35 years; 41.6% males) presenting to the emergency department with acute abdominal pain of less than seven days duration were recruited. The characteristics of the 84 patients are shown in Table 1. Thirty-one healthy controls, matched for mean age and sex were also recruited (median age 32 years, 48.4% male).

Whole Blood LRG1 mRNA and plasma LRG1 in AA diagnosis

Table 2 shows the differentiating features between patients with and patients without AA. Alvarado score and haematemesis were the only discriminating clinical features. Median concentrations of whole blood LRG1 mRNA were significantly different between patients with and patients without AA (1.3 v 2.2 $x10^5$ copies/µl blood; p=0.0134). Median whole blood LRG mRNA normalized to GAPDH was also significantly different between patients with and patients without AA (205 v 371 copies/pg GAPDH; p=0.0004). In addition, median plasma LRG1 protein was higher in AA patients than non-AA patients (54 vs 26 mg/l; p<0.0001).

Figure 1A shows the increase in median LRG1 mRNA concentrations from healthy controls through non-AA, simple AA to complicated AA (Kruskal-Wallis p<0.0001). Figure 1B shows the increase in median LRG mRNA concentrations normalized to GAPDH from healthy controls through non-AA, simple AA to complicated AA (Kruskal-Wallis P=0.0013). There are significant dose-response increases with increasing severity. Figure 1C shows the increase in median plasma LRG1 concentrations from healthy controls through non-AA, simple AA to

complicated AA (Kruskal-Wallis $P<0.0001$). There are significant dose-response increases with increasing severity.

Figures 2 shows the receiver operating characteristic (ROC) curves for LRG1 mRNA concentrations, non-normalized and normalized to GAPDH, plasma LRG1 concentrations, and combination of LRG1 mRNA and protein concentrations in patients with non- versus AA. The area under the curve (AUC) of LRG1 mRNA increased from 0.657 to 0.723 after normalization to GAPDH. When compared to LRG1 mRNA, the plasma LRG1 produced a larger AUC (0.742 vs 0.657). The combination of LRG1 mRNA and plasma LRG1 demonstrated larger AUC (0.743) (Table 3). In Table 3, combination of LRG1 mRNA (normalized to GAPDH) and protein demonstrated the larger AUC (0.781). Combination of LRG1 mRNA (normalized to GAPDH), protein and Alvarado score demonstrated the largest AUC (0.845).

Table 3 shows the add on effect and accuracy of whole blood combinations of LRG1 mRNA, LRG1/GAPDH mRNA, plasma LRG1 protein, and Alvarado score for detecting acute appendicitis. The optimal cut off values for LRG1 mRNA and plasma LRG1 in diagnosis of AA were 2.0 $x10^5$ copies/µl whole blood (sensitivity: 57.5%; specificity: 72.7%) and 31 mg/l (sensitivity: 77.5%; specificity: 68.2%), respectively. The sensitivity of LRG1 mRNA increased to 95% after being normalized to GAPDH (cut off: 188 copies/pg GAPDH). Combination of LRG1/GAPDH mRNA and protein showed the highest sensitivity, which was 97.5%.

Supplemental Figure 1 shows the receiver operating characteristic (ROC) curves for LRG1 mRNA concentrations, non-normalized and normalized to GAPDH, plasma LRG1

concentrations, and combination of LRG1 mRNA and protein concentrations in patients with simple versus complicated AA. The area under the curve (AUC) of LRG1 mRNA, normalized to GAPDH, protein were 0.694, 0.651 and 0.632 respectively. However, combination of LRG1 mRNA and plasma LRG1 did not improve the diagnostic value (AUC: 0.634) in differentiating complicated AA from simple AA. Diagnostic accuracy of whole blood combinations of LRG1 mRNA, LRG1/GAPDH mRNA, plasma LRG1 protein, and Alvarado score for discriminating simple and complicated AA shows on Supplemental Table 1. In differentiation between simple AA and complicated AA, LRG1 mRNA normalized to GAPDH displayed 100% sensitivity and 33.3% specificity. The sensitivity of LRG1 mRNA and mRNA combined with plasma LRG1 were the same, which was 84.6%, whereas the specificity (63% vs 51.9%) and diagnostic value (AUC: 0.694 vs 0.634) of LRG1 mRNA alone were higher.

Factors including LRG1 mRNA, LRG1/GAPDH, LRG1 protein and Alvarado were subjected to multivariate logistic regression. The logistic regression model for discriminating of acute appendicitis and complicated AA are shown in Table 4. Results show that whole blood LRG1/GAPDH mRNA level and Alvarado score are independent predictors of AA. Whole blood LRG1 mRNA and plasma LRG1 protein predict complicated AA.

Discussion

This study shows that normalized and non-normalized whole blood LRG1 mRNA concentrations measured in patients with acute abdominal pain may be used to differentiate patients with acute appendicitis from other causes of acute abdomen, and that the highest levels occur in patients

with complicated gangrenous appendicitis or appendiceal abscess. These findings raise the possibility of LRG1 mRNA as a diagnostic marker.

The diagnosis of acute appendicitis presents a diagnostic challenge to clinicians even when ultrasound and CT are available. Current laboratory diagnostic markers represent a general acute-phase reactant response that is not specific for acute appendicitis [36,37].

The previous discovery that LRG1 protein was elevated in diseased appendices, and also elevated the blood and urine of children with acute appendicitis, even in the presence of negative imaging, raised the possibility of a novel diagnostic marker [7]. Further studies showed that the commercially available LRG1 ELISA was subject to an immunoassay interference effect [8].

Cellular and circulating proteins are downstream biomarkers in pathological processes and as such may represent a late feature in disease processes. Patients presenting with acute conditions require rapid cellular processes to 'switch on' which in turn introduces a delay before biological abnormalities may appear. It is likely that upstream changes in such processes produce molecular changes earlier in acute diseases and may be more useful as early diagnostic and prognostic markers in disease. With this rationale we evaluated changes in LRG mRNA concentrations, the transcriptor for LRG protein, as a potential marker. The performance of LRG1 mRNA for the detection of AA was moderate but nevertheless showed a promising dose-response effect. In addition, present study demonstrated that combination of whole blood LRG1 normalized to GAPDH, and its plasma protein level and Alvarado score improve the diagnostic

accuracy to acute appendicitis, suggesting that LRG1 would have add on effect on Alvarado score in detecting acute appendicitis.

The use of a blood based LRG1 mRNA to enhance current clinical decision rules may improve the accuracy of diagnosing acute appendicitis. An inexpensive but accurate immunoassay could replace the use of advanced imaging and complex RT-qPCR in patients with equivocal clinical presentations. LRG1 mRNA is likely to be elevated in clinical scenarios involving bacterial infections and so its use should be guided by a reasonable clinical suspicion of appendicitis.

This study is preclinical phase study and limited by the time required for RT-qPCR. Nevertheless, appropriate commercialisation would allow the possibility of a point of care test. The study did had a single gold standard for a single condition – acute appendicitis – but it would be important to evaluate the response of LRG1 mRNA in patients with other causes of abdominal pain. Furthermore, LRG1 mRNA offers add on effect on Alvarado score on detecting acute appendicitis. We had to select out samples for study as we had limited funding but ideally all samples from consecutive patients would be analyzed. We have not performed any comparison with Alvarado score, imaging, or other acute phase proteins so it is unclear whether LRG1 mRNA offers any advantage over these markers.

Conclusion

In conclusion, this study shows that both whole blood LRG1 mRNA and plasma LRG1 concentrations are elevated in patients with acute appendicitis and may have a role as a diagnostic marker. A combination of modified whole blood LRG1 mRNA levels, serum LRG1

protein and Alvarado score at the ED may be useful to diagnose AA from other causes of abdominal pain.

325 **Table 1** Characteristics of 84 patients presenting to hospital with acute abdominal pain and
326 suspected acute appendicitis
327

Characteristics	Value
Age	35[16] 18-66
Sex (male,%)	35 (41.7)
Day from symptom onset (day)	2 [3] 1-7
Time of blood collection from arrival of emergency department (h)	11.8 [10.9] 1.7-23.9
Alvarado	6 [3] 2-10
Symptoms (no. of patients, %)	
Nausea/vomiting	38 (45.2)
Haematemesis	0 (0)
Diarrhoea	19 (22.6)
Fresh blood in stool	1 (1.2)
Melaena	1 (1.2)
Abdominal distension	40 (47.6)
Poor appetite	53 (63.1)
Heartburn/Indigestion	7 (8.3)
Change bowel habit	25 (29.8)
Jaundice	0 (0)
Dysuria/urinary frequency	12 (14.3)

Syncope/dizziness	29 (34.5)
Fever	32 (38.1)
Virginal discharge	3 (3.6)
Pain feature	
Tenderness RLQ	84 (100)
Rebound tenderness	35 (41.6)
Migratory RLQ pain	42 (50)
Whole blood parameters	
LRG1 mRNA level (x10^5 copies/µl blood)	1.5 [1.8] 0.24-13.01
LRG1 mRNA level (copies/pg GAPDH)	300 [288] 78-3818
GAPDH (pg/µl blood)	567 [390] 6-1856
Plasma LRG1 protein (mg/l)	39 [40] 4-114
Type of AA (N = 40)	
Simple AA	27 (67.5%)
Complicated AA	13 (32.5%)

All continuous data are expressed as medians [interquartile range] and the whole range.

Numbers may not sum up to 100 because of rounding, multiple factors or absent data

Table 2 Comparisons of factors for discriminating acute appendicitis (AA) and non-AA in 84 patients with abdominal pain and suspected AA

Characteristics	**Non AA (N=44)**	**AA (N=40)**	***p*-value**
Age	36 [14] 18-58	33 [17] 19-66	0.6412
Sex (male,%)	14 (31.8)	21 (52.5)	0.0764
Day from symptom onset (day)	2 [3] 1-7	2 [0] 1-7	0.5424
Time of blood collection from arrival of emergency department (h)	10.8 [12.2] 1.9-23.9	11.8 [9.5] 1.7-22.9	0.5841
Alvarado	5 [3] 2-8	7 [2] 4-10	<0.0001
Symptoms (no. of patients, %)			
Nausea/vomiting	20 (45.5)	18 (45)	1.0000
Haematemesis	0 (0)	0 (0)	0.1013
Diarrhoea	16 (36. 4)	3 (7.5)	0.0151
Fresh blood in stool	1 (2.3)	0 (0)	1.0000
Melaena	1 (2.3)	0 (0)	1.0000
Abdominal distension	22 (50)	18 (45)	0.3453
Poor appetite	26 (59.1)	27 (67.5)	0.5000
Heartburn/Indigestion	5 (11.4)	2 (5)	0.6955
Change bowel habit	13 (29.5)	12 (30)	0.3163

Jaundice	0 (0)	0 (0)	0.1013
Dysuria/urinary frequency	9 (20.5)	3 (7.5)	0.3411
Syncope/dizziness	21 (47.7)	8 (20)	0.0894
Fever	13 (29.5)	19 (47.5)	0.1132
Virginal discharge	3 (6.8)	0 (0)	0.2770
Pain feature			
Tenderness RLQ	44 (100)	40 (100)	0.7434
Rebound tenderness	17 (38.6)	18 (45)	0.6588
Migratory RLQ pain	15 (34.1)	27 (67.5)	0.2446
Whole blood parameters			
LRG1 mRNA level (x10^5 copies/µl blood)µ	1.3 [1.5] 0.3-4.3	2.2 [2.3] 0.2-13	0.0134
LRG1 mRNA level (copies/pg GAPDH)	205 [217] 78-568	371 [232] 149-3818	0.0004
GAPDH (pg/µl blood)	563 [279] 89-1855	591 [615] 6-1503	0.4572
Plasma LRG1 level (mg/l)	26 [38] 4-99	54 [40] 55-114	<0.0001

All continuous data are expressed as medians [interquartile range] and the whole range.

Categorical variables are given as values (percentages).

P values were derived using the Mann–Whitney test or Fisher exact test as appropriate.

Table 3 Add on effect and accuracy (95% CI) of whole blood combinations of LRG1 mRNA, LRG1/GAPDH, plasma LRG1 protein, and Alvarado score for detecting acute appendicitis

	Optimal cut-off	AUC	Improvement in C score*	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Alvarado	>5	0.805 (0.714-0.895)	-	92.5 (79.6-98.4)	56.8 (41.0-71.7)	66.1 (52.2-78.2)	89.3 (71.8-97.7)
LRG1 mRNA ($x10^5$ copies/ul)	>2.0	0.657 (0.538-0.775)	-	57.5 (40.9-73.0)	72.7 (57.2-85.0)	65.7 (47.8-80.9)	65.3 (50.4-78.3)
LRG1/GAPDH mRNA	>188	0.723 (0.614-0.832)	0.066 (10%)	95.0 (83.1-99.4)	47.7 (32.5-63.3)	62.3 (49.0-74.4)	91.3 (72.0-99.0)
LRG1 protein (mg/l)	>31	0.742 (0.635-0.849)	0.085 (13%)	77.5 (61.6-89.2)	68.2 (52.4-81.4)	68.9 (53.4-81.8)	76.9 (60.7-88.9)
LRG1 mRNA + plasma LRG1 protein	>12.4	0.743 (0.636-0.850)	0.086 (13%)	77.5 (61.5-89.2)	68.2 (61.5-89.2)	68.9 (53.4-81.8)	76.9 (60.7-88.9)
LRG1/GAPDH mRNA + LRG1 protein	>1.7	0.781 (0.663-0.879)	0.124 (19%)	97.5 (86.8-99.9)	50 (34.6-65.4)	63.9 (50.6-75.4)	95.7 (78.1-99.9)
Alvarado + LRG1/GAPDH mRNA +LRG1 protein	>5.6	0.845 (0.764-0.925)	0.188 (29%)	87.5 (73.2-95.8)	65.9 (50.1-79.5)	70 (53.4-58.8)	85.3 (68.9-95.1)

* from LRG1 mRNA

Table 4 Logistic regression model of factors for discriminating acute appendicitis (AA) and complicated AA

Factor	Before stepwise		After stepwise	
	Adjusted Odds ratio (95%CI)	***P* value**	**Adjusted Odds ratio (95%CI)**	***P* value**
AA vs non AA				
Whole blood LRG1 mRNA *	0.83 (0.23-2.97)	0.7746		
Whole blood LRG1/GAPDH mRNA *	18.76 (3.27-107.62)	0.0010	16.50 (3.10-87.71)	0.0010
Plasma LRG1 protein *	2.70 (0.74-9.81)	0.1322		
Alvarado	2.00 (1.22-3.13)	0.0052	2.22 (1.46-3.37)	0.0002
Complicated AA vs simple AA				
Whole blood LRG1 mRNA *	7.26 (0.78-66.86)	0.0814	9.72 (1.60-59.12)	0.0136
Whole blood LRG1/GAPDH mRNA *	2.27×10^6	0.9940		
Plasma LRG1 protein *	6.94 (0.85-56.65)	0.0705	5.93 (1.11-31.60)	0.0371
Alvarado	0.81 (0.36-1.85)	0.6220		

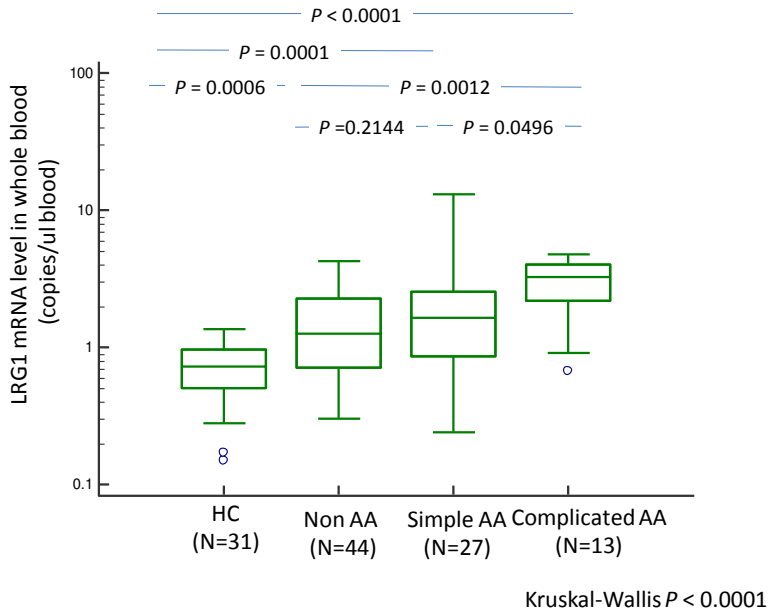
* Optimal cut-off

Figures

Figure 1. Box-plot of median level of (A) whole blood LRG1 mRNA (B) LRG1/GAPDH mRNA and (C) plasma LRG1 protein of healthy controls (HC), non acute appendicitis patients (nonAA), patients with simple AA and patients with complicated AA.

Figure 2 Receiver operating characteristic (ROC) curves of whole blood LRG1 mRNA, LRG1/GAPDH mRNA, plasma LRG1 concentrations, combination of LRG1 mRNA and protein concentrations, combination of LRG1/GAPDH mRNA and LRG1 protein concentrations, and combination of Alvarado (Alv), LRG1/GAPDH mRNA, LRG1 protein concentrations in patients with AA versus non-AA.

Figure 1A



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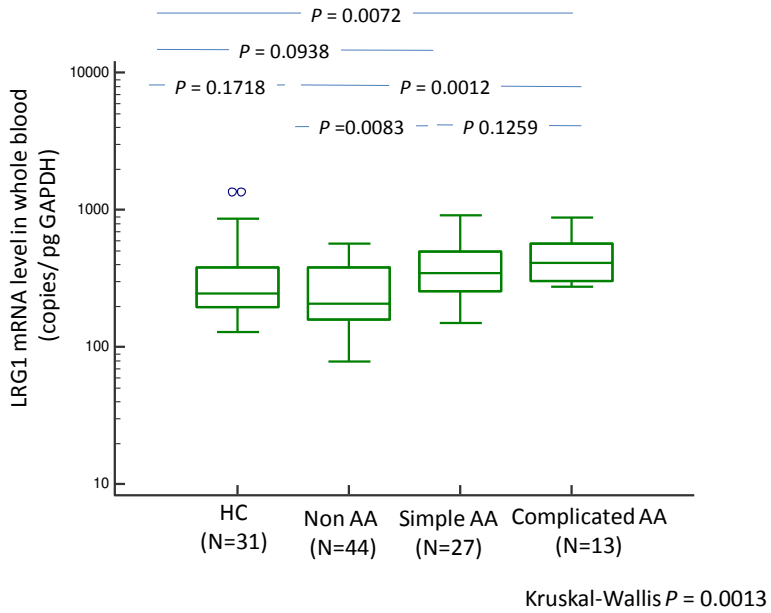
371 **Figure 1B**



372

373 **Figure 1C**

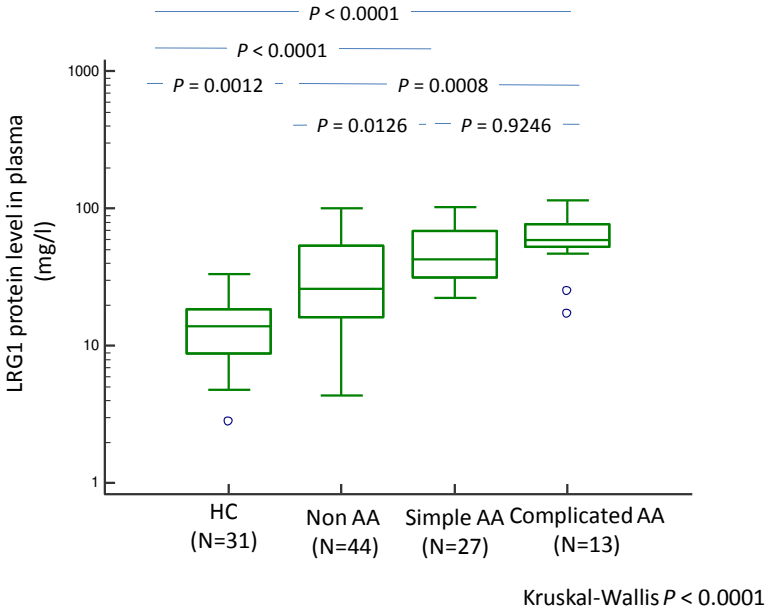


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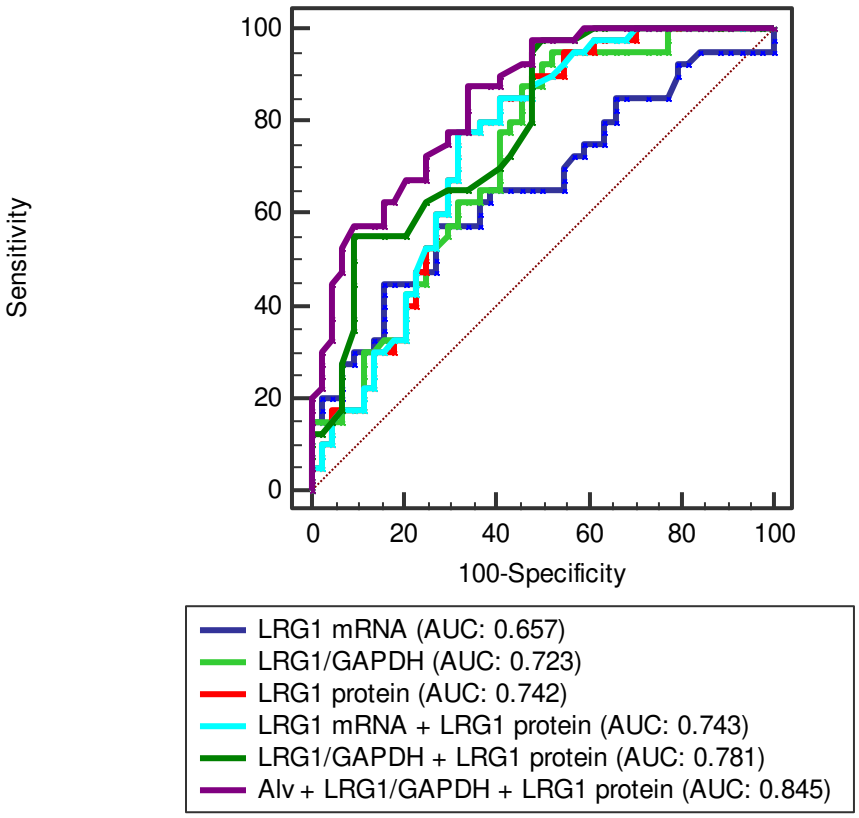
Figure 2


Sensitivity
100
80
60
40
20
0
0
20
40
60
80
100
100-Specificity
LRG1 mRNA (AUC: 0.657)
LRG1/GAPDH (AUC: 0.723)
LRG1 protein (AUC: 0.742)
LRG1 mRNA + LRG1 protein (AUC: 0.743)
LRG1/GAPDH + LRG1 protein (AUC: 0.781)
Alv + LRG1/GAPDH + LRG1 protein (AUC: 0.845)

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Appendix 1

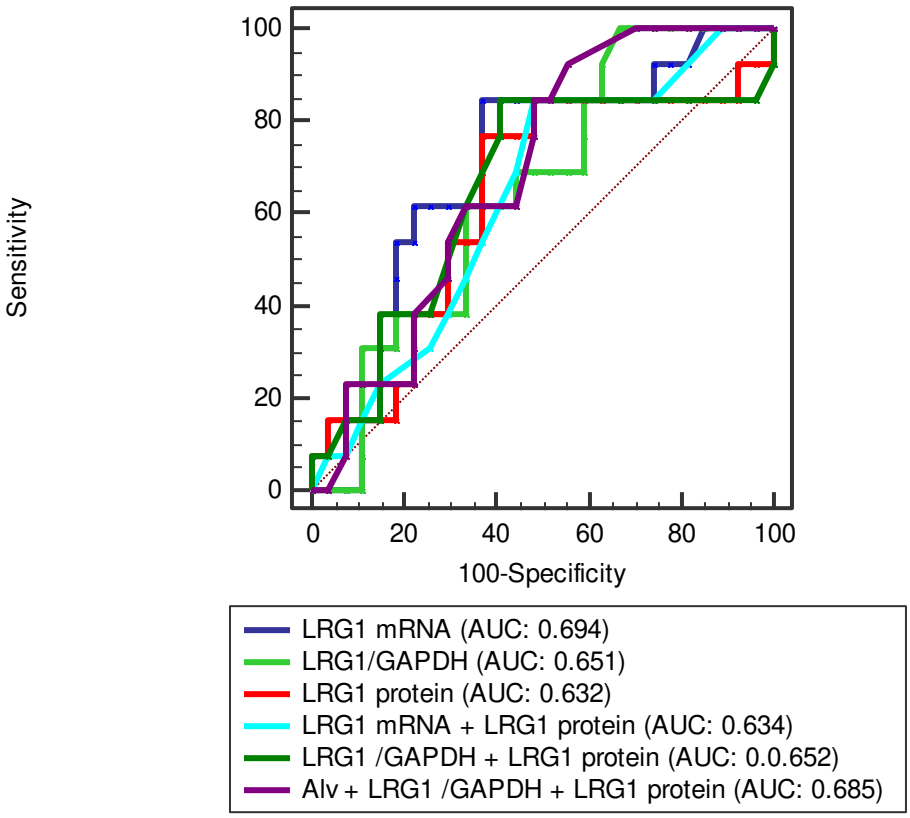
	Sensitivity	Specificity	Accuracy
Plain x-ray	50%	50%	
US (Inexperienced)	75%	86%	80%
ED Physicians	80%	84%	
Alvarado <6	94%	80%	90%
US (Experienced)	90%	100%	96%
CT scan	96 – 100%	95 – 97%	96 – 98%
MRI	100%	94%	

Supplemental Table 1 Accuracy (95% CI) of whole blood LRG1 mRNA, whole blood LRG1 /GPADH mRNA, plasma LRG1 protein, Alvarado, combination of whole blood LRG1 mRNA and plasma LRG1 protein, combination of whole blood LRG1/GAPDH mRNA and plasma LRG1 protein Alvarado, and combination of Alvarado, whole blood LRG1/GAPDH mRNA and plasma LRG1 protein for discriminating complicated acute appendicitis

	Optimal cut-off	AUC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	PLR (%)	NLR (%)
Alvarado	>6	0.655 (0.482-0.828)	84.6 (54.6-98.1)	40.7 (22.4-61.2)	40.7 (22.4-61.2)	84.6 (54.6-98.1)	1.43 (0.97-2.11)	0.38 (0.1-1.46)
LRG1 mRNA ($x10^5$ copies/ul)	>2.1	0.694 (0.518-0.870)	84.6 (54.6-98.1)	63.0 (42.4-80.6)	52.4 (29.8-74.3)	89.5 (66.7-98.7)	2.28 (1.33-3.93)	0.24 (0.07-0.90)
LRG1 /GAPDH mRNA	>261	0.651 (0.477-0.825)	100 (75.3-100)	33.3 (16.5-54.0)	41.9 (24.6-60.9)	100 (66.4-100)	1.50 (1.15-1.96)	0.00
LRG1 protein (mg/l)	>55	0.632 (0.436-0.829)	76.9 (46.2-95.0)	63.0 (42.4-80.6)	50.0 (27.2-72.8)	85.0 (62.1-96.8)	2.08 (1.17-3.69)	0.37 (0.13-1.03)
Whole blood LRG1 mRNA + plasma LRG1 protein	>0.9	0.634 (0.453-0.814)	84.6 (54.6-98.1)	51.9 (31.9-71.3)	45.8 (25.6-67.2)	87.5 (61.7-98.5)	1.76 (1.12-2.77)	0.30 (0.08-1.12)
Whole blood LRG1/GAPDH mRNA + plasma LRG1 protein	>0.7	0.652 (0.453-0.852)	84.6 (54.6-98.1)	59.3 (38.8-77.6)	50 (28.2-71.8)	88.9 (65.3-98.6)	2.08 (1.25-3.46)	0.72 (0.56-0.85)

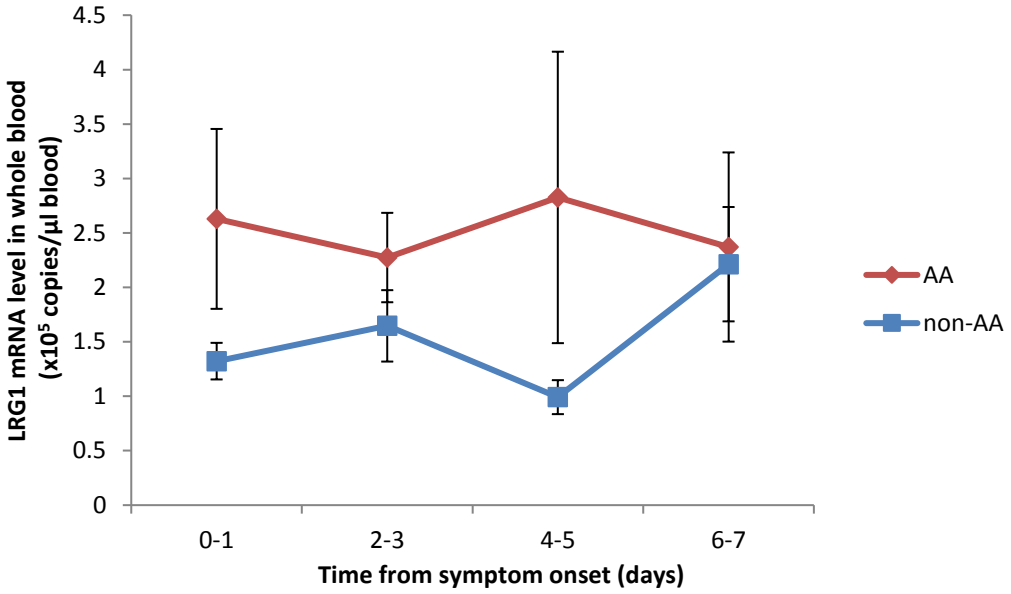
Alvarado + Whole blood LRG1/GAPDH mRNA + plasma LRG1 protein	>3.1	0.685 (0.521-0.850)	92.3 (64.0-99.8)	44.4 (25.5-64.7)	44.4 (25.5-64.7)	92.3 (64.0-99.8)	1.66 (1.15-2.41)	0.68 (0.52-0.82)

Supplemental Figure 1 Receiver operating characteristic (ROC) curves for whole blood LRG1 mRNA concentrations, whole blood LRG1 mRNA normalized to GAPDH, plasma LRG1 concentrations, combination of LRG1 mRNA and protein concentrations, combination of LRG1 mRNA normalized to GAPDH and protein concentrations, and combination of Alvarardo (Alv), LRG1 mRNA normalized to GAPDH, protine concentration in patients simple versus complicated AA

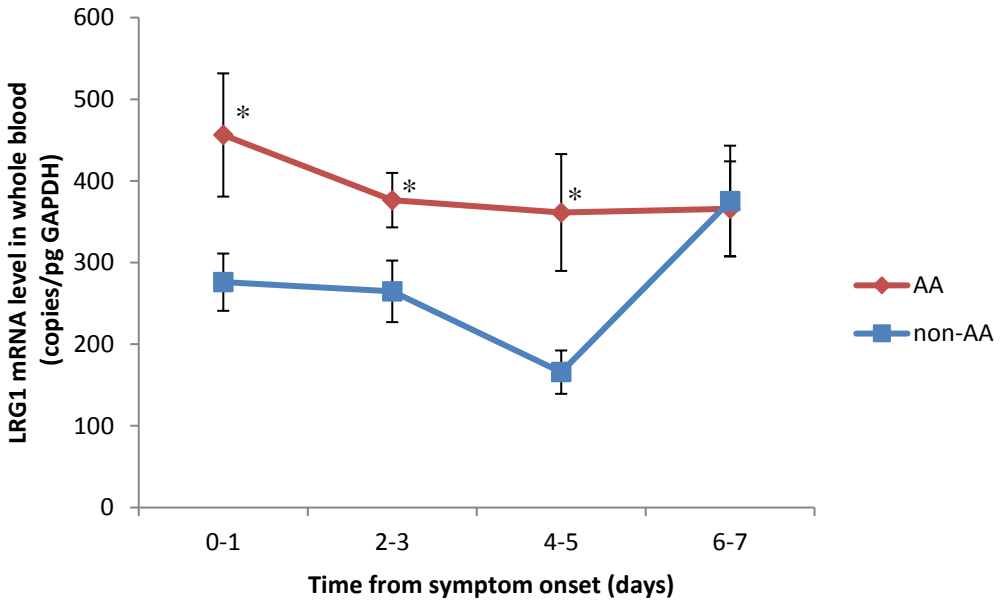


Supplemental Figure 2 Temporal changes in (A) LRG1 mRNA, (B) LRG1 mRNA normalized to GAPDH, (C) and plasma LRG1 from symptom onset (days) to blood sampling for the AA and non-AA groups. Data is presented as the mean ± SEM. Significant difference in LRG1 mRNA or plasma LRG1 was found between AA and non-AA patient with $P<0.05$ by using t-test (*).

Supplemental Figure 2A



Supplemental Figure 2B



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532 Supplemental Figure 2C

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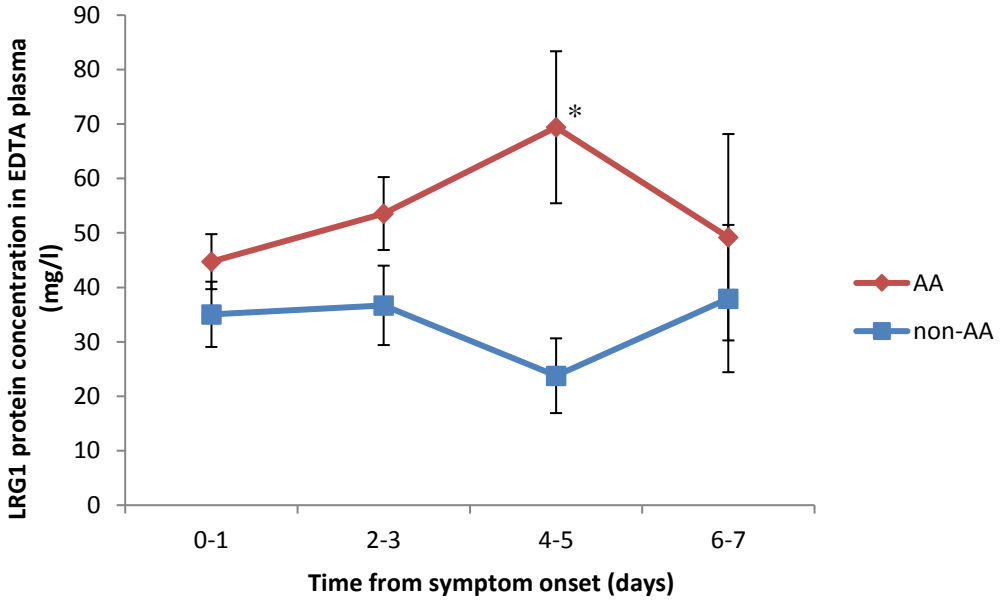

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